NONINVASIVE SCREENING OF NIPPLE ASPIRATES FOR BREAST CANCER-ASSOCIATED BIOMARKERS BY MASS SPECTROMETRY

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Introduction: The incidence of breast cancer is still rising both in the United States and worldwide. While it is hoped that early detection through breast cancer screening will save lives, conflicting observations from screening mammography challenge us to seek more effective screening tools. This study aims to identify protein biomarkers in nipple aspirate (NA) that may assist in detecting breast cancer in the earliest possible stage.

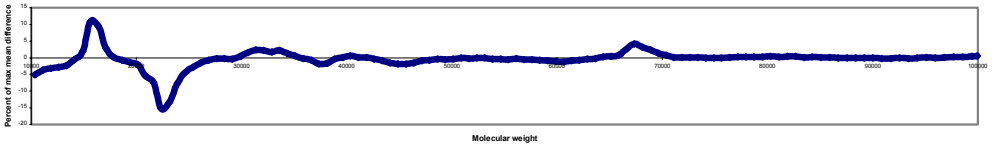
Hypothesis: The protein profile of each NA can predict breast cancer.

Methods: A chip-based surface-enhanced laser desorption/ionization (SELDI) mass spectrometry is being used to identify/count unique proteins in the NA. A semi-quantitative measurement by SELDI allows the comparison of protein profiles from different groups.

To optimize the NA analysis by SELDI, 3 diluants, 3 washing conditions, and 6 types of chips were tested. The condition resulting in the most protein peaks over a large molecular mass range and the most prominent peak intensity is chosen to test all the NA samples.

An SAS application program using repeated measure ANOVA was developed to analyze the SELDI data in the molecular weight ranging between 10,000 and 100,000 daltons. Thirty one specimens were run twice to ensure the interexperimental reproducibility. A total of 57 NA specimens were assayed. Twenty nine were cancerous and 28 were non cancerous. A composite mean profile of NA from the cancerous and non-cancerous groups was generated.

Results and Conclusion: Differences between the composite mean profiles of NA of cancerous and non-cancerous breasts were shown by subtracting mean height values of non-cancerous NA from that of the cancerous NA (Fig)



Eleven protein peaks appear to have different mean levels of abundance between cancerous and non-cancerous NA with a cut off point $p<0.1$: 10370, 11118, 15618, 16368, 20868, 21618, 22368, 23118, 23868, 24618, 67368 daltons respectively.

Our study suggests that protein analysis of NA by SELDI provides statistically significant profiles to differentiated breast cancer from benign conditions. SELDI assay requires minute amount of specimen for analysis, it is efficient and reproducible. It will be useful for large scale screening for breast cancer associated biomarkers.

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GENE EXPRESSION ANALYSIS USING CDNA MICROARRAYS ON SMALL NUMBERS OF CHEMOTACTIC AND INVASIVE CELLS COLLECTED FROM PRIMARY MAMMARY TUMORS

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We have developed metastasis models in rats that are clinically relevant in that they resemble human breast tumors in etiology and histology, permitting real time multiphoton-based imaging of the behavior and interactions of metastatic tumor cells in the primary tumor in vivo. Our previous work has demonstrated that microneedles filled with growth factors and Matrigel, when inserted into the primary tumor, can faithfully mimic the environment that supports invasion and intravasation in vivo. The most efficient cell collection requires the presence of chemotactic cytokines such as EGF and serum components and occurs with 15 fold higher efficiency in metastatic tumors compared to non-metastatic tumors. We have been able to refine the methods necessary for collecting invasive cells from the primary tumor and we can characterize the collection process by direct intravital imaging using the multiphoton microscope. We have been able to position the microneedles in random areas of the tumor allowing a survey of different micro-environments within the tumor. This experimental approach has allowed us to obtain evidence for a paracrine interaction between carcinoma cells and macrophages that increases the invasive potential of the carcinoma cells and the metastatic potential of the tumors.

The fusion of chemotaxis based cell collection with array based gene expression analysis has the potential to identify the genes necessary for individual steps of invasion at the cellular level, and for the rational interpretation of gene expression patterns in metastatic tumors. One drawback to the array technique is the need to isolate and purify microgram amounts of poly(A)+ RNA or around 100 microgram total RNA to generate the appropriate probe. In our study, microneedle collection yields about 1,000 cells containing 30-50 ng of total RNA, well below the amounts needed for arrays. In this study, to validate the use of PCR-amplified full-length cDNA for array based gene expression analysis, SMART cDNA synthesis technology (Clontech) was used. Key to this alternative approach is the reproducible and representative synthesis of cDNA probes that retain faithfully the complexity of the mRNA population present in the original sample. We compared two approaches for synthesizing cDNA probes from total RNA for use with subsequent hybridization to high-density cDNA microarrays: 1) the conventional approach of reverse transcription (RT) of 100µg of total RNA from cultures of carcinoma cells and 2) RT-PCR of ~30ng of total RNA from 1000 carcinoma cells using the SMART system. The results demonstrate that, for situations with limited RNA, the SMART probe synthesis method retains the original mRNA message profile, and is suitable for gene expression profiling of invasive cells collected in microneedles. This technology will allow the characterization of gene expression patterns of tumor cells within the primary tumor during invasion and in response to varying genetic backgrounds. It will also make possible the identification of paracrine and other microenvironment-dependent interactions that contribute to the invasive process.

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FLUORESCENT LABELING METHOD FOR THE DETECTION OF 5-HYDROXYMETHYL 2'-DEOXYURIDINE (HMdU) – A BIOMARKER OF OXIDATIVE DNA DAMAGE

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Increasing evidence implicates oxidative DNA damage as playing an important role in the development of breast as well as other types of cancer. A number of studies have correlated elevated 5-hydroxymethyl-2'-deoxyuridine (HMdU, an oxidized DNA nucleoside) levels with an increased risk of cancer when compared to controls. HMdU has been proposed to be a biomarker of breast cancer risk. We have developed a sensitive fluorometric HPLC method for HMdU quantitation, which should be useful in molecular epidemiology studies.

This fluorescence labeling (FL) method utilizes 7-dimethylamino-coumarin-3-acetic-acid (DMACA) as the fluorescent probe for the pre-column derivatization in anhydrous acetonitrile in the presence of a catalyst at 65^{o}C for 1 h. Thymidine (dT), 2'-deoxyuridine (dU) and and 2'-deoxyadenosine also form fluorescent coumarin products. Reaction with HMdU results in the formation of two baseline separated products. The spectral analysis suggest that dT* (fluorescent labeled dT), dU*, and HMdU* I products are the result of the reaction on the 5'-CH_2OH group on the sugar, while HMdU* II (which elutes later in the normal phase HPLC), is the product of the reaction with 5-CH_2OH on the base. This assignement was confirmed by acid hydrolysis followed by GC/MS analysis.

The fluorescently labeled HMdU, dU and dT products were analyzed using normal phase HPLC (HPLC-2) with fluorescent detection at λ_{ex} = 380 nm and λ_{em} = 460 nm. The quantitation of HMdU*, dU*, and dT* was linear in the range of 5 pmol to 5 femtomol. FL method was optimized for various parameters and validated by taking different amounts of HMdU for the labeling reaction. The limit of quantitation for the HMdU FL method was obtained in the range of 5 pmol to 0.2 pmol. For quantitation of HMdU in DNA, salmon testes DNA or DNA isolated from human white blood cells was enzymatically digested to nucleosides, which are well separated by reverse phase HPLC-1. HPLC fractions corresponding to HMdU were concentrated, subjected to FL, and quantitated using normal phase HPLC-2. During FL analysis of DNA hydrolyzate, we found that an unknown impurity coeluts with HMdU fraction in HPLC-1, which results in an overlaping of that impurity with labeled HMdU on HPLC-2. We are working towards solving this problem. Once the quantitation of HMdU in DNA is achieved, this method will allow to correlate HMdU levels and breast cancer risk.

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THE SEARCH FOR PARAMETERS OF NEGATIVE SLN INDICATORS OF ALN STATUS – A COMPARATIVE STUDY IN BREAST CANCER PATIENTS OF JEWISH ASHKENAZI AND SEPHARDIC ANCESTRY

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In recent years, Sentinel Lymph Node (SLN) has gained wider acceptance as a technique in reducing the morbidity associated with axillary surgery. However, the false negative assessment rate of SLN metastatic involvement is reported as ranging from 0-10% of the patients. Relying on the theory that cancer spreads in an orderly progression from the breast tissue into the sentinel node and then into other nodes in axilla, it is expected that expanding the current pathological test regimen to include new activation and invasion markers (AIMs) might reduce the existing SLN false negative rate.

The aim of this study is to define factors that may improve the sensitivity of SLN investigation, indicating worse prognosis and a high likelihood of non SLN (Axillary Lymph Node (ALN)) metastatic involvement in breast cancer patients.

Three main groups of factors will be evaluated in the primary tumor, SLNs and ALNs of the same breast cancer patient:

- Lymphocyte activation levels following their exposure to tumor cells will be measured mainly in the SLNs and ALNs.
- The primary tumor and its accompanying stroma will be studied for the presence of matrix metalloproteinases (MMPs) and their inhibitors. These are related to degradation of extracellular matrix, an essential step in tumor invasion and metastatic events.
- The presence of chemokines having the capacity to attract epithelial cells to form metastatic deposits will be investigated mainly within the lymph nodes.

The Interactive Transparent Individual Cell Biochip Processor (ITICBP) technology will be used for AIMs measurements.

This methodology is designed to perform a vast spectrum of on-line repeatable measurements, all associated with the same individual cells, group of cells and/or minute biological volumes, while being manipulated and visually observed.

As a result, the combination of static and dynamic parameters of morphometric, fluorometric, chromometric and biochemical measurements of the same individual investigated cell in population, will be available for the first time in pathology.

Monitoring AIMs might, in addition, reveal phenotypic differences between Jewish-Ashkenazi breast cancer patients having a significant percentage of BRCA1, 2 mutations (~25%) vs. the Sephardic breast cancer patients.

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MEASURING IN SITU TELOMERE SHORTENING IN BREAST CANCER

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Normal breast epithelial cells undergo progressive telomeric shortening throughout their replicative lifespan culminating in critically short telomeres, which trigger replicative senescence. Breast cancer cells are able to bypass this growth-arrest mechanism through deregulated expression of telomerase, which maintains telomeres above the minimal critical length. Based on this observation the suitability of these tumors to telomerase inhibitors as direct or adjuvant therapy needs to be determined. To do this we have developed a novel quantitative fluorescence in-situ hybridization system to analyze total nuclear telomeric signal from normal and tumor-derived breast epithelial cells in tissue culture. This technique has been applied to normal human fibroblasts (WI38) successfully and will be applied to two normal Human Mammary Epithelial Cell (HMEC) lines, HME13 and HME 17. Through collection of regular cell and DNA samples we will determine the mean telomere lengths and rate of shortening for each HMEC line and use Q-FISH to determine a per nuclei telomere dynamic. These findings will then be applied to 3-D matrigel models before finally generating predictions of telomere lengths within resected breast tumor samples. This will allow us to determine the suitability of telomerase inhibitors in vivo.

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FLOW CYTOMETRIC ANALYSIS OF ANDROGEN RECEPTOR EXPRESSION IN ARCHIVAL HUMAN BREAST TUMORS

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Hormone receptor expression in human breast tumors is of diagnostic, prognostic and therapeutic value. Immunohistochemistry (IHC) is the standard method used in diagnostic pathology for analysis of these receptors. However, IHC in general is not quantitative and the results are based on visual examination of a small number of cells on a slide. In contrast to IHC, flow cytometry is a rapid quantitative technique for determination of marker expression in human tumor cells. For a variety of technical reasons, flow cytometry has not been developed for routine determination of hormone receptor expression in human solid tumors.

We have recently developed and reported flow cytometric procedures for the analysis of estrogen, progesterone and androgen (AR) receptor expression in human breast and prostate tumors (Clin. Cytometry, 38: 61, 1999; Clin. Can. Res. 6:1922, 2000; Cytometry, 50:25, 2002). Our sample preparatory methods involve enzymatic isolation of nuclei from paraffin sections of formalin fixed tumors, antigen retrieval and staining with anti-hormone receptor antibodies. Propidium iodide is used for staining of DNA and cell cycle analysis of aneuploidy in isolated nuclei.

Earlier studies (Int. J. Can. 52: 581, 1992; J.Path, 170: 31, 1993;Biochem. Biophys. Res. Comm. 2725: 488, 2000) have reported that AR expression is seen in human breast tumors even when estrogen and progesterone receptors are absent. IHC studies have shown that AR expression is seen in 76% of breast tumors and approximately 10% of the AR positive tumors lack estrogen or progesterone receptors. By flow cytometric studies we did not find any discernable AR expression in nuclei from 19/29 of archival formalin-fixed paraffin embedded breast tumors. Positive AR staining was seen in 2/29 tumors with aneuploid subpopulations and 8/29 diploid tumors. The percentage of AR positive nuclei in the aneuploid cells (7.4%) was lower than that of the diploid tumors (21%). Antigen density as measured by comparison of the mean log fluorescence channel (MFC) ratio (of the isotype vs. antibody stained samples), was also lower in the aneuploid tumors than that of the tumors with diploid cells.

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DETECTION OF TUMOR-SPECIFIC DNA IN BREAST CANCER PATIENTS' SERA

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The benefit of early detection of breast cancers is well established. Advances in screening technologies have resulted in a dramatic shift towards diagnosing smaller tumors with improved prognosis. Additionally, lymphatic mapping and sentinel node biopsy has permitted for a more comprehensive evaluation for occult metastasis, which may upstage patients' disease. However, it has been suggested that lymph node metastasis may merely serve as a surrogate marker for systemic disease since hematogenous dissemination can occur in patients without evidence of lymph node involvement. More so, lymph node evaluation can not be performed serially to monitor disease progression. Therefore methods that can identify early systemic progression directly through this blood-borne route may provide a valuable aid to identifying and monitoring patients at increased risk for relapse. We propose molecular detection of tumor-specific circulating DNA in the blood of breast cancer patients may demonstrate utility as a surveillance tool. Blood was collected preoperatively from 63 patients diagnosed with breast cancer (30 patients AJCC stage I, 26 stage II and 7 stage III) and 8 patients during diagnosis of stage IV disease. Serum from each of these patients as well as from 30 healthy female donors was assessed for loss of heterozygosity (LOH) using 8 microsatellite markers on 5 chromosomes (8q, 10p, 14q, 16q, 17q) that frequently demonstrate LOH in primary breast cancers. DNA was isolated and quantitated from 1 ml of acellular serum. Analysis of LOH was performed by gel electrophoresis using a fluorescent scanner. Overall, 28% (20 of 71) of the patients had LOH present in their serum. The incidence of LOH increased with advancing stage: 17% (5 of 30) stage I; 27% (7 of 26) stage II; 43% (3 of 7) stage III; and 63% (5 of 8) stage IV. LOH on chromosome 17 was most common occurring in 7 of 63 patients (11%). LOH was not detected in serum from any of the healthy female donors.

These results reveal the presence of tumor-specific DNA in the blood of breast cancer patients and reflect a correlation to disease stage. This study offers an innovative approach to aid in the assessment of disease progression using molecular analysis of cancer patients' blood.

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CARCINOEMBRYONIC ANTIGEN IN NIPPLE ASPIRATE FLUID AS A MARKER OF MALIGNANCY IN WOMEN WITH MAMMOGRAPHICALLY DETECTED ABNORMALITIES

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Background: Annual mammography combined with physical examination is the current standard for early breast cancer detection. Current methods are however far from perfect. Twenty to twenty-five percent of malignant lesions are not detected with screening mammography and fifty-five to eighty percent of patients who undergo biopsy based upon mammographic findings are diagnosed with benign disease. Carcinoembryonic antigen (CEA) is not produced in the normal breast epithelium but is produced in both pre-invasive and invasive disease. As the early abnormalities that progress to breast cancer first develop within the ductal system, utilizing secretions from the duct to detect CEA is an ideal adjunct to screening mammography.

Methods: Five patients with a demonstrated mammographic abnormality scheduled to undergo biopsy or a definitive surgical procedure were enrolled in study. These included three patients with infiltrating ductal carcinoma (all with associated DCIS), one with papillary cystic carcinoma and one with proliferative breast disease. Nipple aspirate fluid (NAF) was collected from the breast with a mammographic abnormality in all patients and from the contralateral breast in two patients. Diluted NAF (1:50 and 1:100 dilutions in PBS) from all specimens was spotted on nitrocellulose membrane and dried. Purified CEA was spotted as a standard in triplicates in increasing amounts from 0.16ng to 160ng. CEA was detected using the monoclonal antibody T84.66 and a secondary horseradish-peroxidase labeled goat anti-mouse antibody, followed by chemoluminescence detection and 2 min exposure on film. Filters were then stripped and a similar process used to detect the presence of human serum albumin (HSA). Expression of CEA in all tumors was assessed by immunohistochemistry utilizing the same monoclonal antibody.

Results: NAF from the affected breast in all three patients with invasive ductal carcinoma was positive for both the presence of CEA and HSA. NAF from the patient with invasive papillary carcinoma was negative for both CEA and HSA. NAF from the patient with proliferative breast disease was negative for the presence of CEA but positive for the presence of HSA. The contralateral mammographically normal breast was negative for CEA but positive for HSA in the two patients in whom it was collected. Both invasive and non-invasive tumors demonstrated the presence of CEA by immunohistochemistry.

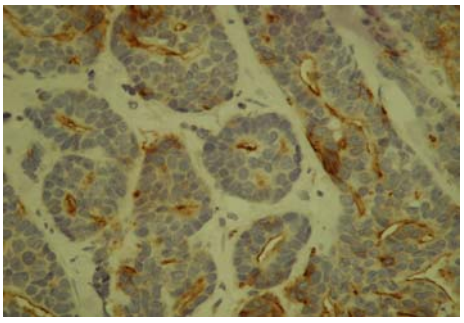

Figure I: Immunohistochemical staining for CEA in infiltrating ductal carcinoma

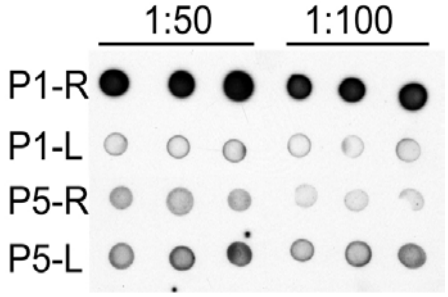

Figure II: Dot blot assay of NAF for CEA

Conclusion: CEA is detectable in the NAF of women with malignant disease of the breast. Detection of CEA may be able to distinguish which patients with mammographic abnormalities will require diagnostic intervention.

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EVALUATING TWO DIFFERENT MINIBODIES, $(SCFV\text{-}CH3)_2$, AGAINST HER2/NEU FOR IMAGING OF BREAST CANCER

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HER2/neu is overexpressed in ~30% of human breast and ovarian cancers. The relatively low expression of this antigen on normal tissues makes this an attractive target. Monoclonal antibodies (mAbs) can be used for delivery of radioisotopes for imaging and therapy. For imaging, intact mAbs (150 kDa) are not suitable as they clear too slowly from the circulation. On the other hand, engineered single-chain Fv (scFv, 27 kDa), consisting only of the variable (V) domains, clears too fast. Intermediate fragments such as minibodies (single-chain Fv-C_H3 fusion proteins, 80 kDa) against carcinoembryonic antigen (CEA) on colon cancer cells have been shown to exhibit rapid, high tumor uptake in xenografts. Moreover, this minibody, labeled with the positron emitting radionuclide, ^{64}Cu ($t_{½}$ = 12.7 h), has shown high-resolution microPET images of xenografts in mice. These results led us to generate and evaluate minibodies against a new target, HER2/neu. In this study we used V genes from two different anti-HER2/neu mAbs, 10H8 [Park J.M. *et al*. Hybridoma 1999, 18:487] and Herceptin. The V genes were assembled into scFv fragments and joined to the third constant domain of human IgG1 ($C_H3_{\gamma 1}$). Constructs were expressed in the mouse myeloma cell line, NS0, at 20-60 μg/ml. Binding to the overexpressing breast cancer cell line, MCF-7/Her, was shown by flow cytometry. Furthermore, the two different minibodies were shown to bind to different epitopes on the antigen by competition. Proteins were purified to >95% purity. Purified protein was conjugated with the chelating agent DOTA and labeled with ^{111}In for biodistribution studies. The 10H8 minibody showed low to moderate tumor uptake and high kidney uptake relatively to the anti-CEA minibody. The Herceptin minibody, evaluated in non-tumor bearing mice, showed similar uptake in the kidneys. This was unexpected, as the size of the minibody is larger than that for the renal filtration threshold (<60 kDa). The Herceptin minibody was also labeled with ^{64}Cu and evaluated in xenografts using a microPET scanner. Again, tumor uptake was low relative to the kidney and liver. Immunohistochemical staining of normal mouse kidneys showed strong specific staining of the proximal tubules. We conclude that the minibodies may be filtered via the kidneys and that they recognize and bind to an antigen in the proximal tubules. Hence, use of anti-HER2/neu fragments for imaging will require further engineering and development.

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OPTICAL TRANSILLUMINATION SPECTROSCOPY AS NONINVASIVE TECHNIQUE TO ESTABLISH BREAST CANCER RISK

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Determining an individual's risk to develop cancer is an important step to a) increase the efficacy of screening procedures by actively recruiting these individuals and b) work with these individuals towards reducing their cancer risk through life style, dietary changes or the use of chemopreventive drugs. Breast cancer risk is currently assessed through interviews, an analysis of the family history or by quantifying parenchymal density pattern as seen on standard x-ray mammography. These parenchymal density patterns reflect the ratio of glandular to adipose tissue within the breast and were shown to provide the highest odds ratio (~6) next to genetic mutations. While the latter affect only a small percentage of the general population, the former affects almost 1/3 providing currently the best independent standard to identify women at risk.

Near-infrared optical transillumination spectroscopy has proven helpful in investigating physiological and anatomical properties of breast tissue, usually in association with optical imaging techniques. Most work to date has focused on exploiting differences in the optical properties between tumor, carcinoma in situ and normal glandular and adipose tissue in the breast. In contrast to this work we hypothesized that the modification in normal breast tissue, giving raise to parenchymal density pattern through differential absorption and scattering of ionizing radiation, will provide also optical contrast and hence a unique optical transillumination spectrum.

The specific aim of the current pilot study is to show equivalence between the optical transillumination spectra and the parenchymal density as breast cancer risk quantifiers. While mammography is an imaging technique only the global density, either as nominal (classification) or interval (%-density) data, is used as risk factor. Similarly the transillumination spectra can be reduced to a set of scalars to establish the respective equivalency, as both techniques are employed in a volume-averaging mode after the respective data reduction.

To date recruited approximately 100 women into this study. Eligibility criteria required negative standard mammograms within the last 12 month. Optical transillumination procedures comprised examination of the breast tissue.

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GENE EXPRESSION CORRELATING WITH CENTROSOME AMPLIFICATION IN BREAST CANCER DEVELOPMENT AND PROGRESSION

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We have developed differential gene expression data sets from breast tumors through analysis of 7 ductal carcinoma in situ, 11 lymph node negative and 12 lymph node positive invasive ductal carcinoma tumors (DCIS, LN- IDC, and LN+ IDC, respectively) relative to 5 normal breast tissues using arrays containing 40,000 DNA elements. 10-20% of these sequences are differentially expressed in breast tumors compared to normal breast tissues. We have taken the novel approach of using centrosome amplification, a tumor-associated phenomenon, to rank tumors so that genes associated with this phenomenon can be identified for further study. Centrosome amplification recently has been identified as a feature common to many human tumors, including breast, and correlates with aneuploidy in breast tumors. Proper function of mitotic centrosomes is required for fidelity in chromosome segregation and preservation of diploidy. Therefore, the identified subset of centrosome amplification-associated genes is likely involved in cell cycle and development of aneuploidy – both of which are key factors in cancer development and progression.

In the present work, centrosome size was quantified by confocal immunofluorescence microscopy and image analysis of normal tissues and the DCIS, LN- IDC, and LN+ IDC tumor tissues. The average values for the tumors (3.06, 2.51, and 3.38, respectively) were all significantly greater than the normal tissue (1.26), but were not significantly different from each other. Therefore increased centrosome size is an early event that occurs prior to invasion and is maintained in most tumors as they invade and progress.

Genes whose expression correlated with centrosome size were identified. DCIS tumors had 66 genes with > 95% correlation with centrosome size. Included are many genes encoding DNA binding proteins and proteins involved with migration and mitosis. Interestingly, LN- IDC and LN+ IDC tumors had fewer genes whose expression correlated with centrosome size, and none of their genes had >95% correlation. LN- IDC tumors had 82 genes with > 80% correlation, while LN+ IDC tumors had only 17. This divergence of gene expression as tumors progress likely reflects the continuing genomic instability perpetuated in part by centrosome amplification.

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THE ROLE OF NUCLEAR βII-TUBULIN IN BREAST CANCER CELLS

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Tubulin, the subunit protein of microtubules, is a major target for anti-tumor drugs, probably because of its role in mitosis. The tubulin molecule is an α/β heterodimer. Both α and β exist as numerous isotypes encoded by different genes. The differences among the β isotypes have been highly conserved in evolution, suggesting that individual isotypes have unique functions. We have found that the β_{II} the isotype of tubulin is unique in that it is localized to the nucleus in a variety of cultured cells, including three breast cancer cell lines: MCF-7, MDA-MB-231 and MDA-MB-435. In samples excised from breast cancer patients, we have found that 20 out of 21 (94%) contained nuclear β_{II}. An interesting finding was that, in metastatic cancer, lymphocytes adjacent to the metastatic cells also contained nuclear β_{II}. In contrast, lymphocytes normally contain no β_{II} at all, either nuclear or cytoplasmic. These results raise the possibility that cancerous cells exert an effect on nearby normal cells which induces them to generate nuclear β_{II}. We have found that nuclear β_{II} is particularly concentrated in nucleoli and that it is in the native state as shown by its ability to bind a fluorescent derivative of colchicine. In order to approach the function of nuclear β_{II}, we have examined the behavior of β_{II} and other isotypes in breast cancer and other cells. We have found that β_{II} interacts with taxol and vinblastine and is expelled from the nuclei of cells. Interestingly, both taxol and vinblastine interact best with the $\alpha\beta_{II}$ dimer, whereas colchicine and nocodazole, that interact best with the $\alpha\beta_{IV}$ dimer, do not expel β_{II} from the nuclei. We have found that breast cancer cells enriched for the β_{III} isotype have elevated levels of free radicals. Since β_{III} lacks an easily oxidized sulfhydryl group, it is not surprising that this should be the case. The sulfhydryl group is present in β_{II}, raising the possibility that β_{II} may be sequestered in the nucleus to protect it from oxidation. We have found, in various cells, that the β_{IV} isotype is associated with actin filaments. However, in MCF-7 breast cancer cells, there appears to be less interaction between β_{IV} and actin, suggesting that the coordination of the different cytoskeletal systems is diminished in these cells. Our results suggest that nuclear β_{II} may have a unique function in breast cancer cells.

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IN VIVO AFFINITY SELECTION AS A METHOD TO ISOLATE ANTIBODY FRAGMENTS AGAINST BREAST TUMORS

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Display of large repertoires of antibody fragments (Fabs) on phage particles has been a tremendous tool in the *in vitro* isolation of antibodies against varied targets such as proteins, carbohydrates and small molecules. The power of this approach is that the isolation of antibodies that bind to a given target occurs by simply allowing the phage antibodies to interact with the target, washing away unbound phage-Fab and then amplifying those that remain. After several rounds of this 'affinity selection', phage antibodies with high affinity to a given target may be isolated. The Fabs themselves may then be expressed and further characterized.

We have produced T47-D human breast tumor xenografts in mice and used the tumor material as the target(s) for the isolation of Fabs from a phage-display library in a similar process, but this time in an *in vivo* setting. In this approach we injected a portion of phage-Fab library, directly into the tail vein of tumor-bearing mice. After a defined period of time the mice were sacrificed by cervical dislocation and their vasculature perfused with saline solution to help eliminate non-binding or loosely binding phage. Tumor material was surgically removed and binding phage isolated and amplified, serving as the injectate for the subsequent round of *in vivo* biopanning. This approach is extremely powerful in that the vasculature of the mouse naturally traps many of the phage-Fab and phage that bind to normal tissues and organs will also be whittled out. Only phage-Fab that travel to the tumor will thus be isolated and thus the screening process is simplified. Furthermore, macromolecular complexes and multi-protein interactions that occur on or within the tumor mass may serve as targets in this *in vivo* setting, something which is much more difficult to examine *in vitro*. Thus without knowing the exact target of interest we may have the ability to isolate antibodies that bind specifically to tumor material.

Here, we report on our initial findings on the *in vivo* biopanning of a phage-Fab display library against T47-D xenografts.

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ENHANCED ENRICHMENT AND DETECTION OF CIRCULATING BREAST CANCER CELLS USING A NOVEL DENSITY GRADIENT SEPARATION MEDIUM AND REAL-TIME RT-PCR

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INTRODUCTION: The molecular detection of circulating tumor cells (CTC) in breast cancer patients has been limited by two factors: 1) low concentration of CTC in peripheral blood, and 2) expression of some cancer-associated genes in normal lymphocytes. In this study, we investigated the ability of a novel buoyant density gradient centrifugation (BDGC) medium (Oncoquick, HEXAL Gentech) to enrich CTC prior to real-time RT-PCR analysis.

METHODS: MDA361 breast cancer cells were spiked into normal PBL by micromanipulation, and processed using Ficoll or BDGC medium. Tumor cell enrichment was assessed with flow cytometry, standard cytology, and real-time RT-PCR.

RESULTS: BDGC medium dramatically reduced the number of recovered mononuclear cells. Molecular analysis confirmed this result with a significant reduction in background expression of CK19 and MUC1. Compared to Ficoll, Oncoquick improved the sensitivity of detection from 1 tumor cell per 105 to 1 tumor cell per 107 mononuclear cells. In a pilot study, 64% of Stage IV breast cancer patients and 100% of those patients naïve to chemotherapy had evidence of overexpression of at least one of five cancer-associated genes in peripheral blood.

CONCLUSIONS: The use of BDGC medium: 1) results in superior reduction in background expression of cancer-associated gene from lymphocytes, 2) enhances tumor cell detection beyond established limits, and 3) has been successfully applied in Stage IV disease. Given the overall efficacy of BDGC medium, its use in Stage I-II disease is promising.

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PRELIMINARY DATA: SERUM FAS AND THE EARLY DETECTION OF BREAST CANCER

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Introduction: With a normal diet, the expression of fatty acid synthase (FAS) in most normal adult human tissues is low. However, cells from a variety of different solid tumor types, including breast tumors, constitutively express FAS. Moreover, FAS expression in pre-cancerous breast tissue at early stages of malignant transformation is also markedly higher than that in normal breast tissue suggesting that activation of the fatty acid synthesis genetic program occurs early in the carcinogenic process. FAS is detectable in human sera with ELISA technology and circulating levels of FAS have been found to be higher among breast cancer patients than controls. However, it is yet to be determined whether pre-diagnostic serum FAS levels predict subsequent development of cancer.

Procedures: We are conducting a nested case-control study of 129 incident breast cancer cases diagnosed from 1990-1998 and an equal number of controls drawn from the CLUE II study cohort of Washington County, Maryland. A third group of 129 women from the cohort who developed benign breast disease during the same time period will also be studied. Stored sera drawn in 1974 and 1989 are being assayed for circulating FAS levels. Absolute, and change over time in, serum FAS levels are being compared across groups.

Results to date: FAS assays have been performed on samples from 50 cases and 50 controls. Mean FAS levels (ng/ml) in 1974 were 12.9 (sd 14.6) and 10.7 (sd 8.0), respectively. For the 1989 samples, mean levels were 17.7 (sd 30.1) and 14.7 (sd 15.7) for cases and controls respectively. The p-values for the paired t-tests of case-control differences were 0.36 for the earlier and 0.53 for the latter samples. The mean change 1974-to-1989 among cases was 7.0 ng/ml (sd 29.5) and among controls was 1.7 (sd 16.4). The p-value for the paired t-test of the case-control difference was 0.28.

Conclusion: Mean 1989 serum FAS levels and 1974-to-1989 serum FAS level change was higher in cases than in controls. However, at the current sample size, these differences were not large enough to attain statistical significance at conventional alpha error tolerances. Moreover, there was substantial overlap of the serum FAS distributions across case and controls. We will have data available on additional case and control subjects by the end of September. The benign breast disease group is now being identified. This study will help determine whether larger-scale evaluations of serum FAS screening for breast cancer are indicated.

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BREAST CANCER TARGETING BY FUNCTIONALLY SELECTED PEPTIDES

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The ability to transfer DNA to specific target cells remains one of the main limitations to the development of gene therapy. To address this problem we are developing selective targeting of neoplastic mammary epithelial cells by combination of microdissection-mediated isolation of pure cell populations with screening for peptide ligands by phage display peptide libraries. Atypical cell populations were morphologically identified in tissues sections of mammary glands collected from TgN(MMTVneu)202Mul virgin female mice and isolated by microdissection from frozen and paraffin sections. Repeated rounds of preabsortion on the normal mammary epithelium and biopanning were performed in order to provide initial selection of peptides bound to atypical but not to normal cells of the mammary gland. Briefly, sections were incubated with blocking solution, phages were applied to the normal mammary gland tissue and unbound fraction was transferred to the microdissected tumor samples. After washing of samples, first fraction of phage was eluted by application of acid elution buffer. The cell debris was saved as a second, tissue-associated fraction. Both fractions were amplified and double precipitated with 20% W/V polyethylene glycol-8000, 2.5 M NaCl. Three to six rounds of biopanning were performed before administration to the animal. To identify peptides with in vivo binding properties total amplified eluates from the last round of biopanning were administered to TgN(MMTVneu)202Mul mice intravenously. After recovery, selected clones were sequenced to determine the consensus sequence. Binding of selected phage clones was determined by distribution of bound phage particles detected by anti-M13 antibodies (Amersham Pharmacia Biotech), and by elutions of selected phage after in vivo administration.

As a result of such an approach a number of peptide ligands have been identified. Their suitability for targeting mammary epithelial cells at specific stages of carcinogenesis in mouse models for breast cancer is currently being evaluated. These experiments shall open new avenues for the rational design of cell-type specific approaches to cancer treatment and prevention.

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MEASURING THE ELASTIC AND HYPERELASTIC PROPERTIES OF BREAST TISSUES

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It is well known that many pathological processes significantly alter tissue elasticity. This has motivated several researchers in imaging communities to develop techniques called elastography to image tissue elasticity. Amongst others, breast elastography has gained a lot of attention, and some researchers have presented clinically promising breast elastography techniques. Interpreting breast elastography images requires knowledge of the linear elastic properties of normal tissues and tumors with known histology results. Furthermore, Finite Element (FE) models developed to predict breast tissue deformation can be used for intra- operative image guidance and image coregistration. In such cases, hyperelastic properties are required. To measure the elastic properties, we use a technique where a small unconfined tissue specimen undergoes indentation while displacement and force data are acquired. If the tissue is linear elastic, the indentation test results in a straight line representing the force vs. displacement. The slope of this line can be converted to the specimen's Young's modulus (E) using a factor that depends on the specimen and indenter geometry and on the boundary conditions. We obtain this factor using FE analysis validated by experiments. To calculate the hyperelastic parameters, we use an optimization algorithm which minimizes the difference between the calculated and observed force vs. displacement curves. Our measurement system, as shown in Figure 1, consists of a load cell for force measurement, a servo actuator with a controller for tissue actuation, and a temperature control system. In our study, fresh tissue specimens coming from mastectomy are dissected into a 15x15x10 mm blocks and undergo indentation experiment at 37^{o}C. In these experiments, a sinusoidal motion with 0.5 mm amplitude and 0.1 Hz frequency is applied. So far, we have tested 30 various breast tissue specimens including normal tissues and tumors. Tumor specimens underwent histology after the experiments and the types of disease were identified. The results are summarized in Table 1. Compared to previous studies, the standard deviation of E is considerably lower which indicates that we have less experimental errors. Furthermore, the stiffness of normal fibroglandular and fatty tissues is almost identical, while carcinoma is ~12 times stiffer. This confirms other studies' conclusion that elastography can be an effective method of breast cancer diagnosis. For hyperelastic properties measurement, the optimization model is undergoing final stage of development. The results of this study will be very useful for the purpose of early and accurate diagnosis of breast cancer.

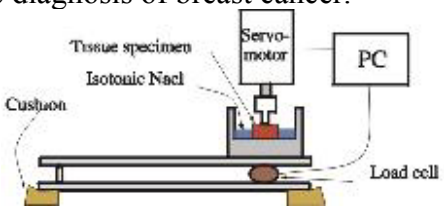

Figure 1: Stiffness measurement system

Fat	Fibroglandular tissue	High grade Carcinoma
2.26±0.44	2.12±0.57	24.75±2.38

Table 1: Young's modulus of breast tissues (kPa)

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DUCTAL LAVAGE AS A TOOL FOR BREAST CANCER EARLY DETECTION

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The current breast cancer diagnostic methods rely on identification of the tumor mass and therefore have limited success for detecting early lesions in which preventive measures could effectively be carried out. We propose a novel approach using ductal lavage technique, coupled with biomarker profile analysis on the fluid and cells obtained. This approach facilitates the detection of malignant or premalignant lesions in individuals who have undetectable tumor masses. We hypothesis that the development of breast cancer is preceded by molecular field disease changes, and therefore cells obtained from any area of the duct will signal the biochemical and molecular changes associated with carcinogenesis. To test this hypothesis, we first performed a molecular mapping study. Ductal epithelial cells obtained from cancereous and non-cancerous ducts were collected from six mastectomy samples. Molecular analysis of proliferation index (Ki67 and PCNA) and methylation of RAR-B were performed. The results showed that significant number of non-canerous duct epithelial cells collected from adjacent fields presented molecular abnormalities. This finding provided direct support for the field disease concept of breast cancer carcinogenesis and further substantiate the ductal lavage technique as an approach for breast cancer early detection. Our next study is to perform ductal lavage on Chinese women who have various risks of developing breast cancer, including patients with newly diagnosed breast cancer, patients with precancerous lesions, healthy female with family history of breast cancerand and heathy female without family history. The rationale to perform this study in Chinese women in China is to test the potential of this technique as an alternative approach for mammogram in detecting breast cancer in third world country. We have just received all IRB approvals from different levels (Chinese Academy of Medical Sciences, UCLA, and DOD) and the study will soon be carried out.

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MOLECULAR DETECTION OF CIRCULATING CANCER CELLS FOR EARLY DIAGNOSIS OF BREAST CANCER

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Early detection is the most significant means for reducing the morbidity and mortality due to breast cancer. Circulating cancer cells may be present in the blood of breast cancer patients at a very early stage of the disease. The goal of this project is to detect circulating cancer cells in the blood of patients who have suspicious mammograms and subsequent breast biopsies using molecular detection of breast-specific tumor markers.

Our molecular detection methods involved: isolation of mononuclear cells and immunomagnetic capture of epithelial cells from 20 microliters of blood using the Dynal System (Dynabeads Epithelial Enrich kit); isolation of mRNA using the mRNA Direct kit and Dynabeads Oligo (dT) 25; reverse transcription of mRNA with random hexamers; specific second strand cDNA synthesis; and real-time PCR with gene-specific primers using an ABI 7700 prism Taqman instrument. Circulating cancer cells were detected using the breast-specific genes, mammaglobin and B726P, as well as gamma-aminobutyrate type A receptor pie subunit (B899P), and cytokeratin-19 (CK-19).

Of the 32 blood samples analyzed to date (anticipated accrual: 200 patients), 13 patients had benign breast disease and 19 patients had invasive breast cancer. CK-19, a marker for epithelial cells, was detected (above the mean copy number from anonymous healthy donors) in the captured epithelial cells from 59% of the 32 patients. Mammaglobin, CK-19, B726P, and B899P were overexpressed (greater than three standard deviations above the mean gene copy number of anonymous donors without known cancers) in the captured epithelial cells from the same 2 patients with benign breast disease. Whereas, mammaglobin transcripts were detected in circulating tumor cells in 74% of the 19 breast cancers. Complementary expression of mammaglobin, B726P and B899P transcripts was detected in 84% of the 19 breast cancers.

In conclusion, our results indicate that circulating tumor cells can be detected at an early stage of breast cancer. Complementation of mammaglobin with additional genes increases the detection of circulating tumor cells. Thus, sensitive molecular detection of cancer cells in the peripheral blood using novel, breast-specific genes can be a beneficial screening test for early stage breast cancer.

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TUMOR-SPECIFIC DNA ALTERNATIONS IN THE PERIPHERAL BLOOD OF WOMEN WITH BREAST CANCER

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We performed a pilot study in subjects with breast cancer to evaluate the hypothesis that detecting occult circulating tumor DNA using a tumor-specific "LOH fingerprint" might have clinical utility. From 14 subjects with 16 primary tumors (Stage IIA or more advanced), we evaluated a total of 30 plasma specimens collected longitudinally during their disease. Multiple foci of each primary tumor, and available nodal or distant metastatic sites, were analyzed with 21 microsatellite markers located at sites of frequent genetic abnormalities in breast cancer. These markers generated an individual LOH fingerprint in 16/16 (100%) tumors; we then looked for this fingerprint in the plasma samples. Mixing studies indicated that LOH could be identified if no more than 25% of the plasma DNA derived from the tumor. LOH in plasma DNA was seen in 20/30 (67%) of plasma samples, from 8/14 (57%) subjects. However, the total # of LOH was small (n = 15), and the mean proportional LOH = 0.05, whereas in tumors it was 0.50. Although they were infrequent, most LOH in plasma DNA (12/15, 80%) were concordant with abnormalities in the subjects' paired tumors, suggesting that they were authentic tumor-derived abnormalities. Despite this, we found no association between LOH in plasma DNA and tumor stage, subject's clinical status at time of blood collection, or clinical outcome. These results suggest that detecting tumor-specific LOH in plasma samples is unlikely to provide diagnostically or prognostically useful information. This may be because the proportions of circulating tumor and normal DNA vary over time and are influenced by multiple factors. More sensitive detection methods, and evaluation of different circulating genetic or cellular abnormalities may be more promising.

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GENES DIFFERENTIALLY EXPRESSED AT THE TRANSITION FROM PREMALIGNANCY TO CARCINOMA

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The goal of this project is to identify genetic differences that exist between atypical ductal hyperplasia (ADH), which are premalignant cancer precursor lesions, and ductal carcinoma in situ (DCIS), the earliest recognized human breast malignancy. To achieve this objective, the project's first aim is to optimize techniques, using murine mammary tissue, to preserve mammary glands, cut and stain sections, identify and microdissect areas of interest (i.e., hyperplasia and cancer), extract RNA and assess its quantity and quality.

Freshly collected normal mammary glands from 12 wk old Fvb mice were immediately fixed in 70% ethanol, 95% ethanol, or Optimal Fix (an ethanol and ethylene glycol containing mix, American Master Tech Scientific). After fixation overnight at 4ºC, glands were paraffin-embedded and 7µ sections were cut and mounted on non-adhesive RNase free slides. Using hematoxylin (H) and eosin (E) stained sections as guides, microdissection of unstained sections was performed using the Laser Capture Microdissection (LCM) apparatus (Arcturus) and RNA was extracted (MicroRNA isolation kit, Stratagene). Tissues preserved with all 3 fixatives generated RNA of good quantity (RiboGreen RNA quantitation kit, Molecular Probes) but poor quality. No 28S or 18S bands were visible on a gel (SYBR Gold nucleic acid gel stain, Molecular Probes), and no PCR products were reproducibly amplified from cDNA generated using this RNA.

Next, similarly collected mammary glands were snap frozen in liquid N2, embedded in OCT (Tissue-Tek, VWR) and stored at -80ºC. 10µ sections were cut, mounted on RNase-free slides kept on dry ice, and returned to -80ºC. After brief fixation in 70% ethanol, sections were stained with dilute H&E (because histology of unstained sections was poor) and RNA extracted as outlined above. We found sufficient quantity and good quality RNA, based on its appearance on a gel and on amplification of 300 bp long products from cDNA generated using this RNA.

In sum, we find that frozen, OCT-embedded mammary tissue, stained with dilute H&E, permits histologic diagnosis and generates good quality RNA after LCM. We will be prospectively collecting and analyzing murine and human mammary tissues using this technique in order to accomplish the project's second aim.

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DEVELOPMENT OF A REVERSE TRANSCRIPTASE CHAIN REACTION METHOD TO QUANTIFY BREAST CANCER CELLS IN THE PERIPHERAL BLOOD

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We have developed a quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) assay to detect small numbers of breast carcinoma cells in the peripheral blood. This assay is based on the observation that breast cancer cells contain cytokeratin 19 (K19) mRNA, but normal peripheral blood cells do not. K19 mRNA was quantified using a 5' → 3' exonuclease (Taqman) real-time PCR assay with β2-microglobulin serving as an internal reference RNA. To evaluate the sensitivity of the assay, SKBR3 breast carcinoma cell RNA was serially diluted in RNA from K562 leukemia cells. The dynamic range of the assay was determined as 0.25 to 400 ng of total SKBR3 cell equivalents of RNA. RNA was then isolated from Ficoll-purified peripheral blood mononuclear cells from normal volunteers or breast cancer subjects with or without one of two methods of enrichment for epithelial cells. Of 17 normal volunteers whose mononuclear cells were not enriched for epithelial cells, K19 mRNA was undetectable in 14 specimens; the mean K19 level in the remaining 3 specimens was 0.29 ± 0.39 ng (SKBR3 RNA equivalents). When blood from 14 normal volunteers was subjected to epithelial cell enrichment using magnetic beads (positive selection), only one volunteer had undetectable levels of K19 mRNA; the mean level for the remaining specimens was 1.84 ± 1.24 ng (SKBR3 RNA equivalents). K19 mRNA was undetectable in 15 Ficoll-purified peripheral blood mononuclear cell specimens from 23 breast cancer patients when no epithelial enrichment was performed; the mean K19 mRNA level in the remainder was 0.58 ± .055 ng (SKBR3 RNA equivalents). When subjected to positive selection, 29 of 29 breast cancer patients had detectable levels of K19 mRNA with a mean of 31.50 ± 160.12 ng (SKBR3 RNA equivalents). [Need RS data] From these data we conclude that we have a sensitive method to quantify circulating breast cancer cells in patients with breast cancer. Subsequent studies are designed to determine whether we can predict disease stage or prognosis using this assay.

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MITOCHONDRIAL MUTATIONS IN TUMORS AND IN NIPPLE ASPIRATE FLUID COLLECTED FROM THE SAME BREAST

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Background: Breast cancer is the leading cause of noncutaneous cancer in U.S. women. Nipple aspiration provides a noninvasive source of breast ductal epithelial cells for the early detection of atypical and malignant changes in the breast. The cells can be screened for mutations in their mitochondrial DNA (mtDNA), which is present at very high levels (1000 to 10,000 per cell).

Hypothesis: That mtDNA mutations which are present in breast tumors can be detected in the NAF of women with breast cancer.

Specific Aims: To determine if mtDNA mutations which are present in breast tumors can be detected in the NAF of women with breast cancer.

Study Design: Screen paired tumor tissue and NAF from women with DCIS or invasive cancer for the presence of mtDNA mutations. 25 mastectomy specimens undergo breast nipple aspiration followed by tumor and normal tissue collection. Both specimens must be satisfactory for mtDNA analysis.

Results: Enrollment: 19 specimens from 18 subjects (one bilateral mastectomy). Prior to using these specimens we analyzed a separate set of matched breast cancer & normal tissue specimens to determine if we could detect both mt deletions and mutations. We determined the sequence integrity of the mtDLP6 region in 23 breast tumors and matched normal adjacent tissue and breast cell lines to evaluate the sequence integrity of the mtDLP6 control element, based upon suggestions that tumors may acquire mutations in their mtDNA genomes that confer a replication advantage driving the mutation to homeoplasty. We hypothesized that these mutations might be good targets for detection in NAFs from different individuals. We observed a higher frequency of C-G transversions at position 431 (4.4 % normal, 21.7 % tumor). We also successfully amplified two mtDNA fragments which span an area of mtDNA which is frequently lost is human disease states, including cancer. We have been routinely successful in amplifying the same area of mtDNA from frozen NAF samples.

Future Plans: In the coming year, we hope to determine if the areas of mtDNA mutation and deletion which are found in breast cancer tissue can also be found in NAF samples, and if so, in the future may serve as a method to noninvasively screen for breast cancer using NAF.

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BREAST CANCER BIOMARKERS IN NIPPLE ASPIRATES FROM WOMEN TREATED WITH ESTROGEN RECEPTOR BLOCKERS

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Background: The breast is the leading site of noncutaneous cancer in U.S. women. Prevention efforts are hindered by the fact that analysis of the tissue at risk has traditionally required a surgical biopsy. Nipple aspiration provides a nonsurgical method of obtaining breast ductal epithelial cells at risk for malignant transformation.

Specific Aims: Determine if 1) the technique of nipple aspiration can be mastered if there is (are) dedicated individual(s) to learn and perform the procedure; 2) candidate biomarkers of breast cancer risk in nipple aspirate fluid (NAF) will change, either favorably or unfavorably, in a subset of women on the STAR (Study of Tamoxifen and Raloxifene) trial; and 3) subjects who have one or more biomarkers which change favorably will have a lower breast cancer risk than those with no change or an unfavorable change.

Study Design: Nipple aspiration at baseline and 6 months after initiating treatment on the STAR trial at centers employing individuals with experience in nipple aspiration. Biomarkers to be evaluated include: cytology, DNA ploidy and cell cycle parameters using image analysis, and prostate-specific antigen (PSA). We will evaluate the success in obtaining a nipple aspirate sample and determine if biomarker changes developing after treatment with an estrogen receptor blocker were associated with histologic deterioration in the breast to atypia or cancer.

Results: 25 subjects have enrolled thus far. NAF has been successfully collected from 24/25 subjects at 33/34 visits. Cytologic analysis has been performed on samples from 22 participants, with hyperplasia observed in 4 women at baseline. No woman has demonstrated hyperplasia after 6 months of treatment, although results are only available for 8 women thus far. Other biomarker results are pending.

Future Plans: 1) continue to accrue subjects and perform biomarker evaluation; and 2) longterm follow-up of subjects to determine if biomarker changes are associated with risk of future breast cancer.

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BREAST CANCER AND BONE MINERAL DENSITY

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Recent studies have shown that women with low bone mineral density (BMD) have a low risk for breast cancer. Therefore, it has been suggested that mammography may not be worthwhile for older women with low bone density. Measuring BMD at age 65 and stopping mammography in women who have low BMD has been proposed as a cost-effective clinical practice. However, women with newly diagnosed breast cancer have not been evaluated to determine what their BMD levels are at the time of diagnosis. The purpose of our study is to assess the BMD of women 65 years of age and older with newly diagnosed breast cancer in comparison with the bone mineral density of same aged women with normal mammograms and to examine the risk factors associated with breast cancer and low bone mass in these two groups of women.; 3) to develop a model based on the study population to determine the predictive value of low bone mass for risk of breast cancer.

We are in the process of recruiting women 65 years and older for 150 cases, women with within 4 months of their definitive surgical procedure for breast cancer, and 150 controls, women within 4 months of a normal mammogram. At one clinic visit, subjects complete a health questionnaire. Height, weight, waist and hip girth are measured. Bone mineral density is measured at the hip, spine, forearm, and total body by dual energy x-ray absorptiometry (Hologic QDR 2000).

Preliminary results from 24 cases and 42 controls were evaluated. The mean age for both groups is 72 years. Bone mass index is higher in cases than controls, 27.1 (±4.1 SD) versus 26.2 (±6.0 SD). Bone mineral density at the total hip was lower in the cases in comparison with the controls, 0.785 g/cm2 (±0.108 SD) and 0.795 (±0.127 SD), respectively. At the lumbar spine, the mean BMD was also lower in the cases, 0.933 (±0.126 SD), than controls, 0.978 (±0.182 SD).

In the first group of women evaluated for this study, the BMD of women with newly diagnosed breast cancer is lower than controls. However, the results of this study are preliminary and cannot be yet be used to make any conclusions.

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AN EPIDERMAL BIOSENSOR FOR CARCINOEMBRYONIC ANTIGEN

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Early detection of breast cancer is critical to successful management, treatment and cure. The purpose of our research is to develop a new approach to early detection of breast cancer – an epidermal biosensor. This concept is based on the hypothesis that keratinocytes can be genetically engineered to act as an in vivo biosensor. An epidermal biosensor would be a small area of skin (<0.5cm in diameter) containing keratinocytes genetically modified to recognize and respond to molecules secreted into the circulation by a tumor. As a prototype, we proposed to develop an epidermal biosensor to carcinoembryonic antigen (CEA) as an early, sensitive, continuous in vivo monitor to signal onset of breast cancer.

We are testing new cell-surface receptors designed to bind and respond to CEA. The chimeric receptor has an extracellular ligand binding domain based on a single-chain recombinant antibody (scFv) to CEA. The intracellular response domains are based on the EGF receptor or the TNF-α receptor. The research focuses on three experimental goals:

1. construct a plasmid containing the sequence for a chimeric cell-surface receptor that would bind CEA and initiate an intracellular response.
2. introduce the construct into human keratinocytes in vitro and select for cells expressing the chimeric receptor.
3. test the binding and response of the genetically modified keratinocytes to CEA.

Our progress can be summarized as follows:

1. Chimeric receptor - scFv anti-CEA Fc γ: Generation of retroviral vector; introduction and expression into human keratinocytes and HaCaT cells in vitro; demonstration of binding of I-125-CEA to scFv anti-CEA-Fcγ keratinocytes.
2. Chimeric receptor - scFv anti-CEA TNFαRI: Construction of plasmid and generation of a retroviral vector; introduction and expression into human keratinocytes and HaCaT cells; absence of CEA binding.
3. Chimeric receptor – scFv anti-CEA EGFR: Construction of plasmid and generation of retroviral vector; introduction and expression into human keratinocytes and HaCaT cells; ongoing experiments are aimed at measuring binding of I-125 CEA to scFv anti-CEA-EGFR HaCaT cells and evaluating activity of cells in response to CEA.

We have thus far concluded that human keratinocytes can be genetically altered to express chimeric receptors in vitro. One of these chimeric receptors has been shown to bind CEA. We plan to continue our research to learn if keratinocytes expressing a chimeric receptor that recognizes CEA can be engineered with a receptor that will give a cellular response in the presence of CEA. We then hope to test these altered keratinocytes in an in vivo system as the basis for an epidermal biosensor for CEA.

The long-term objective is to explore the use of epidermal biosensors as a continuous, *in vivo* monitors for the presence of tumor antigens expressed by breast cancer. The expectation is that epidermal biosensors could provide early detection of the onset of disease for high-risk patients so that appropriate medical management could be initiated when it is most likely to result in a positive outcome.

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EARLY DETECTION OF BREAST CANCER BY MOLECULAR ANALYSIS OF DUCTAL LAVAGE FLUID

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Detected early, breast cancer is an eminently curable disease. However, reliable intermediate biological markers of risk, easily detectable in both pre-and post-menopausal women, do not exist at the present time. Mammography is a powerful detection technique. But there is clearly a need for developing new methods for early detection of breast cancer. In this study, we have tested the application of methylated genes as markers for detecting breast cancer cells in ductal lavage fluid of high risk women. The branching ductal system of the breast converges at 5-8 openings on the nipple surface. Since most breast cancers arise from the ductal epithelium, atypical and malignant cells can be collected in breast ductal fluid. By performing a genome-wide search for genes that are differentially expressed in breast cancer, we identified genes that had lower expression in breast cancers and were silenced by hypermethylation of promoter sequences. Three hypermethylated genes, Cyclin D2, RAR-β and Twist were selected because they were each hypermethylated in more than 30% of breast cancers, and unmethylated in normal mammary epithelial cells, mammary stroma and white blood cells (WBC). We extended the analysis to 56 DLF from patients at high risk for breast cancer (Gail index>=1.7) with normal breast exam and mammograms. By cytology, 50 samples were benign or with mild atypia and 6 samples were markedly atypical. Methylated alleles were detected in 4 of 6 atypical DLF samples. Interestingly, 2 women with positive MSP and marked atypia by cytology were diagnosed with breast cancer, and a third patient is undergoing evaluation. Only 5 of 45 cytologically benign samples were MSP positive. In combination with cytology evaluation, MSP of ductal lavage fluid could provide a useful adjunct for the early diagnosis of breast cancer (Lancet, April, 2001). In an effort to increase the sensitivity and specificity of detection of cancer cells in ductal lavage fluid, we have now added two more frequently methylated genes, RASSF1A and HIN-1 to the panel. By MSP, one or more hypermethylated genes were detected in each of the invasive ductal (41/41) and lobular (20/20) carcinomas tested. Methylated alleles of the five genes were also frequently detected in DCIS (49/51, 96%) and in LCIS (12/17, 71%). However, analysis of multiple markers is limited by the small number of cancer cells retrieved from the ductal lavage fluid. To circumvent this problem, we have developed a nested, multiplex PCR approach whereby all five genes are amplified using the same aliquot of DNA. The sensitivity of this technique is increased by at least 100-1000-fold compared to direct MSP. Using this approach, we will now examine ductal lavage fluid obtained from 25 women with breast prior to surgery, and 25 women at high risk for developing breast cancer. The development of a panel of 5 markers that detect 100% invasive carcinoma and nearly all DCIS, combined with a reliable method for retrieving cells from the ducts are very important steps to the final development of a "PAP-test" for the breast. They will provide the ability, not only to detect breast cancer early, but also to provide reliable intermediate markers for testing novel modalities of prevention and therapy, all three of which have important implications for breast cancer research and to all women.

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LOSS OF THE TIGHT JUNCTION PROTEIN CLAUDIN-7 CORRELATES WITH HISTOLOGICAL GRADE IN BOTH DUCTAL CARCINOMA IN SITU AND INVASIVE DUCTAL CARCINOMA OF THE BREAST

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Despite improvements in breast cancer therapy, the survival rates of women with metastatic breast cancer remain very low. The loss of cell-to-cell cohesive forces is known to be a central factor in the process of tumor cell dissemination and metastasis. Claudins are a recently discovered family of 20 transmembrane proteins responsible for the sealing of tight junctions, which play a major role in maintaining cell-to-cell adhesion in epithelial cell sheets. However, their role in cancer progression remains largely unexplored. First identified by us through SAGE analysis, we have confirmed that Claudin-7 expression is lost in invasive ductal carcinomas (IDC) of the breast by both RT-PCR (9 of 10) and Western analysis (6 of 8). Immunohistochemical (IHC) analysis of increasing histological grades of ductal carcinoma in situ (DCIS) and IDC showed that the loss of Claudin-7 expression correlated with histological grade in both DCIS ($p < 0.001$, $n = 38$) and IDC ($p = 0.014$, $n = 31$), occurring predominantly in high-grade (Nuclear and Elston grade 3) lesions. Tissue microarray analysis of 355 IDC cases confirmed the negative correlation between Claudin-7 expression and histological grade ($p = 0.03$) found in case by case analysis. This pattern of expression is consistent with the biological function of Claudin-7, as greater discohesion is typically observed in high-grade lesions. This correlation was further strengthened by the observation that by IHC analysis Claudin-7 expression was lost in the vast majority (13/17) of lobular carcinoma in situ lesions, which typically display a discohesive structure. In summary, these studies provide insight into the potential role of Claudin-7 in breast cancer progression and suggest that Claudin-7 may be valuable as a prognostic indicator for breast cancer.

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INTRADUCTAL ABLATION OR DIFFERENTIATION OF MAMMARY EPITHELIUM AS A MODE OF PREVENTION OF BREAST CANCER

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Advances in molecular medicine have lead to increased identification of women with an elevated breast cancer risk. As a novel preventive strategy we tested the hypothesis that selective elimination or differentiation of epithelial cells lining the ductal network by administration of cytotoxic agents through the ductal network would prevent breast cancer. To test this concept, we tested the efficacy of 1) a targeted killing by nonreplicating adenovirus AdHTK, 2) replicating, lytic vaccinia virus (vaccinia-hemagglutinin viral vector) and, 3) the SERM, 4-hydroxytamoxifen (4-OHT), in the prevention of nitrosomethylurea-induced rat mammary tumors. We first demonstrated the efficacy of killing of two rat mammary cell lines RBA and NMU38, and a human mammary epithelial cell line, HBL 100 following infection with the two viral vectors in culture. Infection fo the cultured cells with AdHTk, followed by addition of gancyclovir to the cultures resulted in death of the cells in a dose dependent manner. Infection with vaccinia vector resulted in rapid death of all the cells within 48 h. By wholemount analysis of the treated glands, we observed that injection of the Vaccinia-HA constructs into rat mammary glands led to the destruction of more than 80% of the ductal system. In rats administered vaccinia through the teat, a single injection of 100,000 virus particles resulted in significantly delaying latency of mammary tumor development in the rats. Intraductal injection of ADHTK followed by gancyclovir treatment resulted in a significant reduction in tumor incidence compared to untreated rats. In contrast to the vaccinia treated rats, destruction of the epithelial network was barely visible. Injection of 4-OHT (50ug/duct) at weekly intervals for 6-8 weeks in 8 to 10 of the 12 glands per rat resulted in the development of 1 in 222 treated glands, compared to control animals (64 tumors in 288 glands). These results demonstrate the feasibility of the intraductal approach in accessing all the ductal cells in the mammary gland. Further, they illustrate the potential application of the intraductal approach to breast cancer prevention, and possibly therapy by localizing the toxic effects of the agents to the duct bearing the tumor.

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IDENTIFICATION OF POTENTIAL BIOMARKERS FOR THE EARLY DIAGNOSIS OF BREAST CANCER

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Breast cancer is one of the leading causes of cancer mortality in woman. The successful treatment of the disease depends on early diagnosis. Currently the diagnostic tools for breast cancer are limited, and often suffer from drawbacks. For example, mammography can miss small lesions, and sometimes can induce tumors in certain patients. Tests of biopsied tumor samples can only be performed in symptomatic patients when the tumor has been identified. Certain blood-borne markers have been used in affordable, routine serum tests to diagnose cancer in asympotamatic patients. However, these markers, when used individually, can detect the presence of only a certain percentage, but not all, of a particular type of tumors. This argues that a panel of blood-borne markers should be identified and used for the diagnosis of each type of cancer. It is conceivable that multiple blood-borne markers, when used in combination, could predict the existence of tumors more accurately and more successfully. Once the presence of a tumor is determined, cell surface markers can be used in imaging analysis to identify the location of the lesion.

We propose to systematically isolate secreted and cell surface proteins (trafficked proteins) with increased expression in early stage breast tumors. These proteins are candidates for blood-borne markers and cell surface markers that can be used in the routine screening of early stage breast cancer. We will first isolate all secreted and cell surface proteins from breast tumors of multiple patients, using a functional approach we have designed and validated. Next, the expression levels of these proteins in normal and early stage breast tumor tissues will be compared, and those with increased expression in tumors will be identified and analyzed. These studies will generate a pool of potential biomarkers, which can be evaluated through further studies for their use in the early diagnosis of breast cancer.

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CYTOGENETIC EVIDENCE THAT CIRCULATING EPITHELIAL CELLS IN PATIENTS WITH CARCINOMA ARE MALIGNANT

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Numerous studies of circulating epithelial cells (CEC) have been described in cancer patients and genetic abnormalities have been well documented. However, with one exception in colorectal cancer, there has been no attempt to match the genetic abnormalities in the CEC with the primary tumor. The purpose of this investigation was to determine (1) whether CEC in patients including those with early tumors are aneusomic and (2) whether their aneusomic patterns match those from the primary tumor indicating common clonality.

Thirty cancer patients had CEC identified by immunofluorescent staining using a monoclonal anti-cytokeratin antibody. Their CEC were analyzed by enumerator DNA-probes for chromosomes 1, 3, 4, 7, 8, 11 or 17 by dual or tricolor-fluorescence *in situ* hybridization. Touch preparations of the primary tumor tissue were available from 16 of 30 patients and were hybridized with the same set of probes used to genotype the CEC.

The number of CECs from each patient ranged from 1 to 92 cells per cytospin. CEC showed abnormal copy numbers for at least one of the probes in 24 of 30 patients. Touch preparations from the primary tumors of 14 patients with aneusomic CEC were available. The pattern of aneusomy matched a clone in the primary tumor in 10 patients.

We conclude that the vast majority of CEC in breast cancer patients are aneusomic and are derived from the primary tumor. Therefore, excess CEC are candidates for early detection, immunophenotyping and genotyping, for recurrences to guide targeted-therapy and to help prognostication.

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RT-PCR DETECTION OF BREAST CANCER METASTASES IN SENTINEL LYMPH NODES OF BREAST CANCER PATIENTS

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Sentinel lymphadenectomy, a minimally invasive surgical procedure, has been widely adopted as standard-of-care for the management of breast cancer (BrCa) patients in the hands of experienced surgeons. Current methods to detect cancer metastases fail to identify a large number of patients with metastatic disease. The hypothesis of this study is that analysis of sentinel lymph nodes (SLN) by reverse-transcriptase polymerase chain reaction (RT-PCR) for specific markers may more accurately detect metastases and predict recurrence than routine histological methods. We have previously identified mammaglobin (MAM) and carcinoembryonic antigen (CEA) as exceptional molecular markers for the detection of occult disease. Here we report the results to date of PCR analysis of SLN from patients enrolled in our multicenter trial.

Methods: 794 patients were enrolled in an IRB-approved multicenter trial. SLN were localized by dual injection of isosulfan blue dye and ^{99}Tc sulfur colloid. Alternate serial sections of SLN were designated for pathology (H&E ± pan-cytokeratin IHC) or RT-PCR. PCR was performed using primers for mammaglobin (MAM) (40 cycles) and/or carcinoembryonic antigen (CEA) (32 cycles plus Southern Blot). Reactions were performed at least twice using two cDNA preparations and stringent controls.

Results and Conclusions: PCR was performed on blinded specimens from 209 patients. 99% (93/94) of tumors tested were MAM positive and 94% (62/66) were CEA positive. 33% (68/209) of patients had histology positive SLN, consistent with clinical expectations. PCR identified MAM transcripts in 94% of these histology positive patients; only 2 patients failed to express either MAM or CEA. Each marker increased the detection of occult metastases. MAM PCR detected SLN metastases in 42% of histology negative patients. With the addition of CEA, 48% of histology negative women are upstaged. These data correlate with clinical observations that 30-40% of BrCa patients with histology-negative LN still recur and die of their disease. At 3.8 yrs follow-up, a greater number of MAM PCR-positive patients have recurred than MAM PCR-negative patients (11 vs. 4). Patients with histology negative LN who are upstaged by MAM PCR have a decreased recurrence-free survival relative to patients with histology negative/PCR negative SLN (cumulative survivals = 72.3% vs. 97.4%; percent censored = 94.9% vs. 97.6%). Although these differences do not reach statistical significance, they support the hypothesis that MAM PCR detection of micrometastases is a useful prognostic indicator of BrCa recurrence. More accurate detection of SLN metastases may distinguish women truly at low risk for recurrence from those patients most likely to recur and to benefit from aggressive therapy.

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INDICATIONS AND CONTRAINDICATIONS OF SENTINEL NODE BIOPSY

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Sentinel node biopsy (SNB) is replacing axillary node dissection as the standard of care for patients with breast cancer to determine the presence or absence of lymphatic metastases.

METHODS: A multi-center trial was initiated in 1997 to systematically determine those factors that influence the false negative and identification rate of SNB in breast cancer patients. We have previously elucidated surgical experience as a critical factor for successful SNB and have helped establish national credentialing guidelines. Over 1200 patients have been enrolled in this on-going clinical trial. The injection technique in the majority of patients was peritumoral and/or intradermal using both technetium sulfur colloid and isosulfan blue dye. A small group of patients were also injected with peri-areolar fluorescein. All marketed gamma probes were used intraoperatively to detect the sentinel nodes. The factors analyzed have included the impact of preoperative chemotherapy, prior lumpectomy, presence of multi-focal and multicenteric disease and performance of sentinel node biopsy in the presence of ductal carcinoma in situ.

RESULTS: In a group of 780 patients who underwent complete axillary node dissection following sentinel node biopsy, 53 (7%) were found to have multicentric/multifocal disease and were compared to those with single site disease. Neither the false negative rate, identification rate, nor the number of sentinel nodes obtained differed significantly. A small group of 26 patients received preoperative chemotherapy and a sentinel node was identified in 96% (25/26) with no false negative results. A total of 49 patients had pure DCIS with no evidence of invasive disease on final pathology and 2/49 (4%) were found to have a positive sentinel node. In 117 patients who received injections of all three agents (isosulfan blue dye, technetium sulfur colloid and fluorescein dye) the majority of the positive sentinel nodes were blue (76%), hot (90%) and fluorescent (84%). A re-analysis of our false negative patients showed a higher false negative rate (11%) in the patients that have undergone a prior partial mastectomy compared to those that have had either a prior open biopsy of core biopsy (4%, $p<.02$). Molecular staging of 38 false negative sentinel nodes, in 19 patients, using RT-PCR for mammoglobin found that 55% (21/38) were actually positive. By comparison, RT-PCR analysis on 41 histologically negative non-sentinel nodes found only 7% (3/41) to be positive. In all of the patients with histologically negative sentinel nodes, that were negative for mammaglobin by RT-PCR, the surgeon had done less than 30 SNB procedures.

CONCLUSIONS: This study shows that SNB is accurate in patients with preoperative chemotherapy or multifocal/multicentric disease. Sentinel node biopsy can also identify a small group of DCIS patients that may benefit from more aggressive therapy. The actual false negative rate of sentinel node biopsy is lower when using more sensitive methods of detecting metastatic disease. Our data continues to widen the applicability of the technique as well as identify those factors that require further investigation.

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ELECTROCHEMICAL DNA BIOSENSORS FOR DECENTRALIZED BREAST CANCER SCREENING

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The use of DNA testing as an important component of breast cancer diagnosis has been increasing rapidly during the 1990s. The goal of this research is to develop, optimize, and test a miniaturized sensing system for the rapid and reliable genetic screening detection of breast-cancer. In accordance to our original goal our studies have focused on various fundamental and practical aspects of electrical detection of DNA segments specific to the breast-cancer gene BRCA1.

Electrochemical devices have received considerable attention in the development of sequence-specific DNA hybridization biosensors. Such devices rely on the conversion of the DNA base-pair recognition event into a useful electrical signal. The high sensitivity of such devices, coupled to their compatibility with modern microfabrication technologies, portability, low cost (disposability), minimal power requirements, and independence of sample turbidity or optical pathway, make them excellent candidates for centralized and decentralized DNA diagnostics. During the first part of this project we introduced new and innovative electrical strategies and routes for improving the reliability of devices for genetic screening of breast-cancer. In particular, we have successfully combined the unique amplification features of new metal nanoparticles or enzyme tags, with an effective magnetic separation (isolation) of the duplex, and a powerful electrical detection for achieving the task of selective and sensitive breast-cancer screening. Such developments address the challenges of mismatch discrimination, signal amplification, non-specific adsorbates, and should facilitate the realization of instant point-of-care breast-cancer testing.

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DEVELOPING REAGENTS FOR MUTATION ANALYSIS USING PHAGE DISPLAY

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The goal of this work is to devise a method for mutation analysis. The concept is based on the fact that peptides can adapt to any molecular surface with high affinity. Thus, peptides could be selected that will recognize point mutated DNA. We have prepared a phage display library and used this to screen for reagents that bind ssDNA. Several phage were identified which suggests that this approach is feasible. This method will now be applied to the generation of reagents that recognize dsDNA and point mutated DNA. We employed the fd-Tet vector using a cyclic or constrained variable region where the disulfide bridges formed by cysteines cyclize this portion. The oligonucleotide that we have inserted encodes for the amino acid sequence A-E-C-X_{10}-C, where the A-E would be the amino terminus of pIII following cleavage and the X represents random amino acids generated through random nucleotides at these positions. The insert also contains Apa1 and SpeI restriction enzyme sites flanking the inserted region. The inserts had the following sequence: Apa1-TGT-N30 -TGT-Spe1 (where Apa1 and Spe1 represent the recognition site). The library was then characterized which showed a complexity of $5x10^8$ unique phage and that 98% contained an insert.

To isolate and characterize phage that recognize ssDNA, 5 mg of biotinylated oligonucleotides were bound to 60 mm neutravidin coated plastic dishes overnight and then washed. Phage stock with a titer of 1 x 10^8 that had been pre-cleared on neutravidin coated plates were incubated with these plates overnight at 4 C. The plates were then washed 10 times, followed by elution of bound phage with 0.1 N HCL, pH 2.2. The eluates were concentrated, dialyzed against TBS, precipitated using PEG/NaCl and then resuspended in 50 µl of TBS. This was then used to infect K91 cells and phage were harvested. These phage were then used to screen dishes with the biotinylated oligonucleotides bound. A total of four rounds of enrichment were used. As controls throughout this process, phage with no insert were used, as well as plates that had neutravidin but no oligonucleotides bound. After the first round, both the no/neut and lig/neut plates screening showed mainly random sequences along with one clone that appeared to be ~40% of the sequences present. However, by the fourth biopan, there was a distinct evolution towards related sequences. In the no/neut plates, one clone predominated. In the lig/neut plates, one clone was 50% of the sequences, and interestingly it was related to some of the other clones isolated. Two other clones showed similarity to each other. These clones are unrelated to the no/neut clones and did not predominate in the early rounds of screening suggesting that indeed there was some specificity to the selection. Further work will now be performed to confirm that these clones do indeed recognize ssDNA.

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REGULATION OF THE ACTIVITY OF MITOTIC CENTROMERE-ASSOCIATED KINESIN (MCAK)

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Mitotic centromere-associated kinesin (MCAK) is a protein which is required for the proper segregation of chromosomes during cell division. Overexpression of MCAK disrupts microtubules and leads to aberrant mitotic spindle assembly. Depletion of MCAK protein levels leads to lagging chromosomes during anaphase and aneuploidy. MCAK has been identified as a protein which shows high levels of expression in proliferative breast cancer tumors.

Rather than transporting cargo along assembled microtubule polymer like most kinesin family proteins, MCAK depolymerizes microtubules. In this way MCAK influences the dynamic behavior of microtubules in the cell. Some chemotherapeutic drugs may also function, in part, to alter the dynamic properties of microtubules. Because MCAK is required to ensure the correct ploidy in daughter cells during cell division and also because MCAK influences the dynamic behavior of microtubules it is essential to determine how MCAK activity in the cell is regulated.

We have identified three types of MCAK regulation that occurs in mammalian cells; (i) structural regulation, (ii) posttranslational regulation and (iii) tubulin-linked regulation. In the first case we have determined that the final five amino acids at the extreme C-terminus of the protein inhibits the microtubule depolymerizing activity of MCAK. Premature truncation or other alterations in C-term. structure could produce overactive MCAK protein which would adversely affect cellular microtubules. In the second case, we have linked MCAK to the ubiquitin conjugating enzyme system. Posttranslational modification of MCAK by ubiquitin may target the protein for degradation or for localization to specific regions of the cell. Finally, we have found that the tubulin autoregulatory system responds to the level of MCAK protein in the cell. The level of tubulin polymer in the cell determines how much tubulin dimer is synthesized by that cell so that the ratio of polymer to dimer remains constant. We have found that when the level of MCAK changes within the cell, the tubulin autoregulatory system responds accordingly. This suggests that MCAK levels are critical for microtubule function and indicates that MCAK may be a suitable marker for early stages of cancer or as a target for cancer therapy.

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DETECTION OF MUTATIONS USING A NOVEL ENDONUCLEASE

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We discovered a nuclease, CEL I from celery, that has high specificity for DNA mismatch, including base-substitutions, insertions and deletions. It has been used to develop a robust mutation detection assay. We have optimized this assay, and demonstrated its utility in the screening of mutations and polymorphisms of BRCA1, ARSC, MED1, and TDG genes, of hundreds of individuals. Multiple mutations in one PCR fragment can be simultaneously identified. The assay has been adopted for high throughput screening of EMS induced mutations in plant genomes to facilitate plant genetics studies, and for the detection of mutations in large genomic regions of microorganisms. Current effort is aimed at enabling this assay for the genomic scanning of mutations in higher organisms, the detection of mutations in pathogens such as Bacillus anthracis, and early cancer detection.

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DEVELOPMENT OF A MICROARRAY ELISA FOR MEASURING CANCER BIOMARKERS

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We have developed a sandwich microarray ELISA for the detection of low-abundance protein markers of breast cancer. We initially tested this microarray ELISA using hepatocyte growth factor (HGF). We were able to quantitatively detect HGF with detection limits at the sub-pg/mL level. Using this HGF microassay, the mean HGF concentration in sera of breast cancer patients (680 pg/mL; range 200-1600 pg/mL) was found to be significantly elevated compared to control sera (390 pg/mL; range 150-1000 pg/mL), in agreement with a previous report. The serum HGF levels measured with the microarray ELISA results correlated ($r2 = 0.90$) with values obtained using a commercial 96-well ELISA assay. Therefore, these data demonstrate that the microassay is suitable for analysis of clinical samples. In multiplex studies, we simultaneously assayed 5 potential markers at biologically relevant levels. These studies indicated that excellent signal-to-background ratios could be obtained for each assay even though the concentrations of the different antigens varied 3000-fold. All of the reagents and detection equipment required to perform the assay are commercially available and this technology could be readily utilized by laboratories currently using fluorescent cDNA microarray technology and also should be transferable to a clinical setting. Therefore, this technology represents a sensitive, quantitative, and readily available approach to high-throughput protein analysis. Although our initial studies used serum, we expect the microarray technology to be particularly useful in the evaluation of small volume samples such as biopsies or nipple aspirate fluids. In summary, we have developed a new technology to evaluate levels of specific proteins in bodily fluids or tissue samples that may aid in predicting the presence, stage or recurrence of breast cancer.

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